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AGE AND SEX ASSOCIATED DIFFERENCES IN THE RELATIVE ABUNDANCE OF MULTIPLE SPECIES OF CYTOCHROME P-450 IN RAT LIVER MICROSOMES.
---A SEPARATION BY HPLC OF HEPATIC MICROSOMAL CYTOCHROME P-450 SPECIES --

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Age-associated alterations in drug metabolism have been observed in experimental animals by many investigators (1-6) and has been interpreted in terms of human geriatric pharmacology. However, we have shown that age- and sex-associated differences in drug metabolism are phenomena that are not clearly separable from each other (1). In that study, a complete disappearance of sex difference in some of the monooxygenase activities was demonstrated in old age, while the activities were more than several fold higher in young males than in young females (1). We proposed that the age associated alterations in relative abundance of multiple species of cytochrome P-450 in male rat liver microsomes were the major cause of these old age-associated alterations in activity. This was based upon our observations of peak-height ratios of ethylisocyanide difference spectra (1,7) which altered with age, male values becoming closer to female values in old age. In the present study we employed the HPLC technique to semiquantitatively visualize the differences of relative abundance of multiple species of cytochrome P-450. This method was first successfully employed by Kotake & Funae (8) in 1981, and was shown to be useful for demonstrating alteration in the relative quantities of cytochrome P-450 species.

MATERIALS AND METHODS

Chemicals NADP, NADPH, NADH were obtained from Oriental Chemicals Inc. (Japan). Glucose-6-phosphatedehydrogenase and cytochrome c (type III) were obtained from Sigma Chemical Co. (USA).
Animals and preparation of solubilized liver microsomes Male and female rats of Fischer-344 strain were purchased at the age of 4 weeks from Japan Charles River Co. (Atsugi) and were kept in the institute's aging colony under SPF conditions. Fifty percent survival times for these animals were 27 months for males and 30 months for females.
Liver microsomes were prepared according to the Omura and Sato method (9) with the exception that the final microsomal pellet was homogenized and suspended in double distilled water. The microsomal preparation was then solubilized with sodium cholate in the presence of 20 % glycerol according to the method used by Warner et al. (10).
HPLC elution and analysis of solubilized microsomes A Shimadzu HPLC system LC4A equipped with a gradient programmer capable of mixing three different solvents and an anion exchange column (Farmacia MonoQ) was used for the analysis of solubilized microsomes. The absorption of the protein was monitored at 417 nm. Mobile phase A (buffer A) was a 5 mM tris-acetate buffer containing 0.2 % Emulgen 913 (Kao Atlas) and 20 % (v/v) glycerol adjusted to pH 7.2. The chromatogram was developed with a 93l-min multi-step gradient of 1 M sodium acetate in 20 mM trisacetate buffer containing the same concentration of Emulgen 913 and glycerol (buffer B) after 30-min run with 100 % buffer A. The flow rate was 0.25 ml/min. The solubilized microsomal preparation (2.5 mg protein) was applied to the column after passing through a 0.45 um millipore filter and sephadex GX-25 column to remove sodium deoxycholate used for solubilization. The experiment was performed at 20-23 °C. The eluent fractions with high 417 nm absorption were applied to SDS-polyacrylamidegel electrophoresis (11) for molecular weight analysis. Carbon monoxide difference spectra (9) were also recorded for these fractions.
Assay-method Microsomal protein concentrations were determined by the method used by Lowry et al. (12). Cytochrome P-450 contents in liver microsomes were determined spectrophoto-

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metrically using a Hitachi UV-340 spectrophotometer (9). Aminopyrine N-demethylase (AND)(13), hexobarbital hydroxylase (HBH)(14), aniline hydroxylase (AH)(15), p-nitroanisole O-demethylase (p-NAD)(16), 7-ethoxycoumarin O-deethylase (ECD)(17), lidocaine N-deethylase (LND)(18) and imipramine N-demethylase (IND)(19) activities were assayed according to the previously described methods, as indicated.

RESULTS and DISCUSSION

As indicated in Table 1, there were marked sex differences in AND, HBH, LND, IND and ECD activities in young animals, but significant sex differences no longer existed in old age due to drastic decrease in activities in male liver microsomes. Sex differences in AH and p-NAD activities were small or nonexistent even in young animals.

As shown in Fig. 1, there are at least 40 insufficiently separated peaks eluted from the HPLC column, which is a great improvement in separation as compared to the previous publication (8). Not all of these 417 nm absorbing species in solubilized microsomes represent cytochrome P-450 species. Also, each of these peaks does not necessarily represent a single protein species, but most contain electrophoretically heterogeneous proteins. Some of these peaks do not contain proteins of molecular weight which corresponds to that of P-450 as determined by SDS-polyacrylamidegel electrophoresis. However, most of the peaks from 1 through 27 contain proteins of molecular weight corresponding to that of P-450 in high concentrations and show the CO difference spectra characteristic of cytochrome P-450. The peaks 8, 11, 14, 16, 17 and 19 are induced by phenobarbital with 11 as the major peak. The peaks 17, 18 and 19 were induced by 3-methylcholanthrene with 19 as the major peak (data not shown). A distinct sex difference is present in the elution patterns of solubilized liver microsomes in young animals. In 3 and 12 month old rats, the area under the elution curve in the region indicated by M (AUC_M) is larger in male than in female rats, while that in the region indicated

TABLE I. Sex Differences in Hepatic Microsomal Cytochrome P-450 Dependent Drug Metabolizing Enzyme Activities in 3, 12 and 24-month-old Rats

	MALE			FEMALE		
	3 months	12 months	24 months	3 months	12 months	24 months
Cytochrome P-450 (nmol/mg)	0.677 ± 0.045	0.700 ± 0.058	0.484** ± 0.041	0.569 ± 0.027	0.553 ± 0.035	0.504 ± 0.029
AND	6.55°° ± 0.202	5.74°° ± 0.319	2.44** ± 0.149	2.31 ± 0.050	2.95 ± 0.269	2.40 ± 0.191
HBH	2.04°° ± 0.138	1.94°° ± 0.140	0.244** ± 0.013	0.245 ± 0.003	0.274 ± 0.029	0.283 ± 0.058
LND	4.95°° ± 0.249	2.68**°° ± 0.082	0.158** ± 0.021	0.153 ± 0.025	0.116 ± 0.011	0.136 ± 0.016
IND	6.31°° ± 0.872	6.18°° ± 0.690	2.27** ± 0.375	2.02 ± 0.080	1.84 ± 0.096	2.05 ± 0.043
ECD	0.314°° ± 0.025	0.309°° ± 0.031	0.247* ± 0.016	0.245 ± 0.017	0.271 ± 0.029	0.238 ± 0.020
AH	1.37 ± 0.046	1.21 ± 0.062	0.936** ± 0.064	1.16 ± 0.053	1.18 ± 0.076	1.05 ± 0.067
p-NAD	0.848 ± 0.059	0.933 ± 0.027	0.852 ± 0.066	1.13 ± 0.042	1.14 ± 0.027	0.912* ± 0.081

°° Significantly higher than correnspondingfemale values ($p<0.01$)

*, ** Significantly smaller than the values for 3-month-old rats of corresponding sex. (* $P<0.05$, ** $P<0.01$).

Values are means ± S.E. of activities of 6 individually prepared liver microsomes.

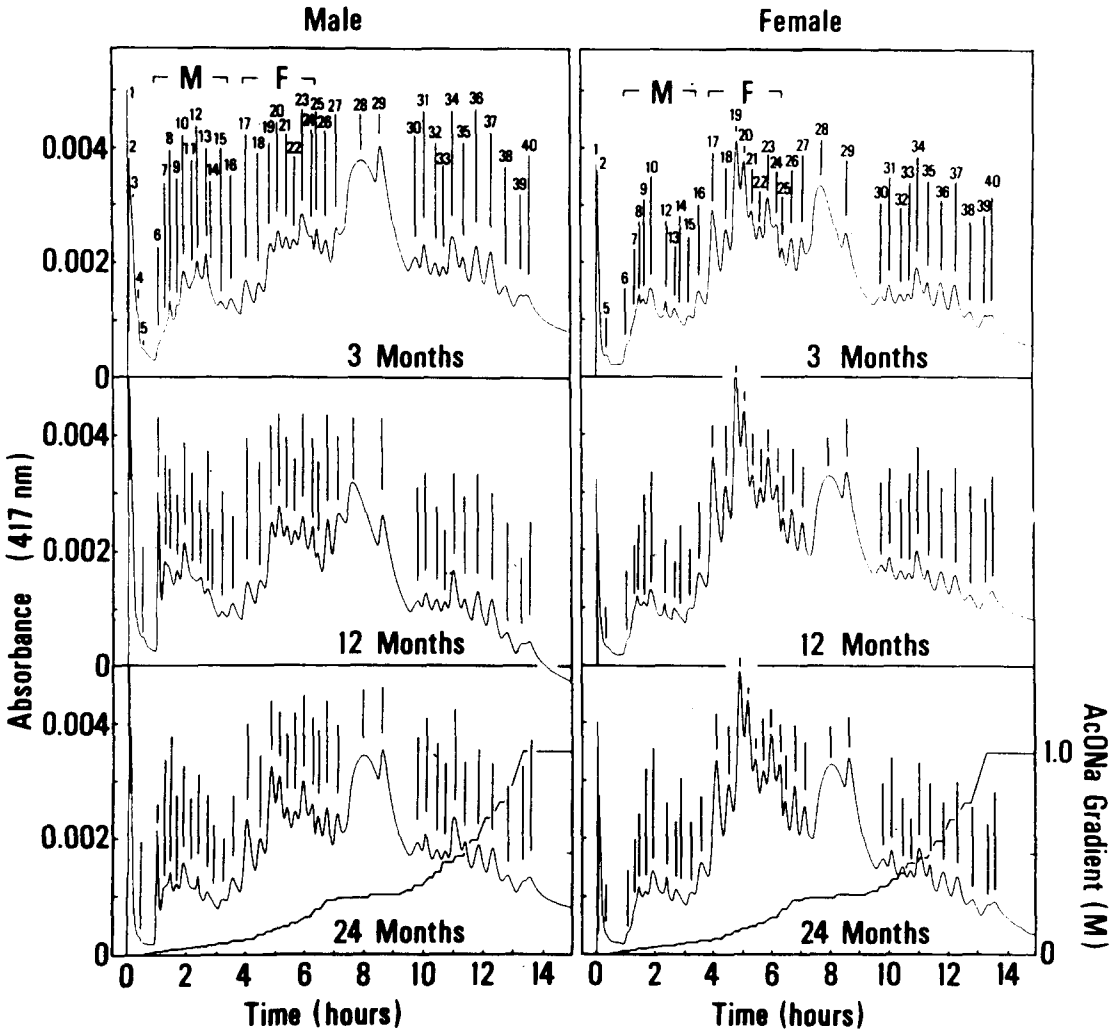

Fig. 1. HPLC elution profiles of solubilized liver microsomes from 3, 12, and 24-month-old male and female rats. The chromatogram was developed with a stepwise sodium acetate gradient program from 0 to 1 M as indicated in the lower most panel of the figure.

by F (AUC_F) is larger in female than in male rats. The ratio between AUC_M and AUC_F in males was 0.61±0.03 (mean±S.E.) and in females, 0.37±0.03. It seems that the male predominant species of P-450 are localized in region M and the female predominant species, in region F. To be more spcific, in 3-month-old males, the peaks 10, 12, and 13 in region M were higher than those of females, while in females, peaks 17, 18, 19 and 20 in region F were distinctively higher than those of young males. Furthermore, in females, peak 17 is distinctively higher than 18, and 19 than 20, while in young males, peaks 17 and 18 are low and of almost the same height, and 20 is higher than 19. In addition to these sex differences, peak 6 is markedly high in 12 and 24-month old males. The 3-month-old males and females did not show this characteristic peak. However, none of the monooxygenase activities tested corresponded to this species (TABLE I), indicating that another pattern of age-associated alterations in monoxygenase activity exists. Kamataki et al. (20) recently reported the presence of at least two sex specific P-450 species in young rat liver microsomes. Our results are in fair agreement with their results, since both found different P-450 species predominant in male or female rats. More studies are necessary, however, to identify which of the features observed in the present study corresponds to the sex specific species reported in their study.

A distinct age difference in the elution pattern was also found in male rats, while in females age difference was essentially nonexistent. The only exception was a further increase in

heights of the peaks with old age in region F which was already higher in young females. The AUC_M to AUC_F ratio in old males decreased to 0.46±0.02 approaching the young female values. The value also decreased in old females to 0.3±0.04. Furthermore, in old male rats, peaks 10, 12 and 13 in region M which are higher in young males than females decreased to female levels and the peaks 17 and 19 in region F which were higher in females increased, resulting in a pattern resembling that of females with the exception of peak 6. The fact that P-450 species present in higher concentrations in young males than in young females decreases with age to the female level may explain the disappearance of sex differences in monooxygenase activities in old age (TABLE I). Interestingly, in collaboration with our laboratory, Kamataki et al. also observed the loss of male specific P-450 in liver microsomes from old males using an immunological quantitation. Thus the composition of sex specific P-450 species in male liver microsomes became similar to that of female liver microsomes at 24 months of age (21). The monooxygenase activities which did not show much of a sex difference in young animals did not decrease with age. These activities may probably be associated with the P-450 species which do not decrease with age. These results suggest that the age-associated substrate selective alterations in drug metabolism are caused by the age-associated alterations in relative abundance of multiple species of cytochrome P-450. It appears that the most drastic alteration is the decrease in P-450 species which are responsible for the sex differences in drug metabolism.

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